

# Differential Regulation of Lipogenesis and Leptin Production by Independent Signaling Pathways and Rosiglitazone During Human Adipocyte Differentiation

Nayan G. Patel,<sup>1</sup> Julie C. Holder,<sup>2</sup> Stephen A. Smith,<sup>2</sup> Sudesh Kumar,<sup>1</sup> and Margaret C. Eggo<sup>1</sup>

Since leptin levels are independently correlated with risk of coronary heart disease, we have identified signaling pathways important in mediating leptin production and lipogenesis in human preadipocytes. We used inhibitors of p70<sup>S6</sup> kinase, p42/44 mitogen-activated protein kinase (MAPK), p38 MAPK, and phosphatidylinositol 3-kinase (PI3K). Human preadipocytes were induced to differentiate in insulin, dexamethasone, triiodothyronine, and 3-isobutyl-1-methylxanthine in the presence or absence of inhibitors and the peroxisome proliferator-activated receptor (PPAR)- $\gamma$  activator rosiglitazone. Differentiation was assessed by measuring leptin secretion, lipid content, and lipogenic activity. Rosiglitazone increased cell protein by 15%, the lipid content of the cell layer was doubled, and the lipogenic activity increased sevenfold but did not stimulate leptin secretion. None of the inhibitors significantly inhibited protein content over 20 days, but lipid content and lipogenic activity were inhibited by p70<sup>S6</sup> kinase and p38 MAPK inhibition but not by p42/44 MAPK or PI3K inhibition. All of the inhibitors significantly decreased leptin secretion, and these inhibitory effects were increased by coinubation with rosiglitazone. We conclude that PI3K and p42/44 MAPK pathways are not critical to the differentiation program leading to lipid accumulation, but stimulation of leptin secretion is dependent on these as well as the p70<sup>S6</sup> kinase and p38 MAPK signaling pathways. *Diabetes* 52:43–50, 2003

**H**uman obesity is a significant risk factor in a number of diseases, such as non-insulin-dependent diabetes (type 2 diabetes) and hypertension (1). Obesity is characterized by an increase in the size of lipid stores in adipocytes and an increase in the number of adipocytes. These are derived from a pool

From the <sup>1</sup>Division of Medical Sciences, University of Birmingham, Birmingham, U.K.; and <sup>2</sup>GlaxoSmithKline, Harlow, Essex, U.K.

Address correspondence and reprint requests to Dr. Margaret C. Eggo, The Medical School, Birmingham, B15 2TT, U.K. E-mail: m.c.eggo@bham.ac.uk.

Received for publication 6 June 2002 and accepted in revised form 23 September 2002.

N.G.P. and S.K. have received grant/research support from GlaxoSmithKline. J.C.H. and S.A.S. are employed by and hold stock in GlaxoSmithKline.

C/EBP, CCAAT/enhancer-binding protein; Dex, dexamethasone; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; G3PDH, glycerol-3-phosphate dehydrogenase; FKBP12, FK-506-binding protein-12; HBSS, Hank's balanced salt solution; HRP, horseradish peroxidase; IBMX, 3-isobutyl-1-methylxanthine; LPL, lipoprotein lipase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; T<sub>3</sub>, triiodothyronine; TBS-T, Tris-buffered saline-Tween 20; TNF, tumor necrosis factor.

of existing preadipocytes, which are ready to differentiate when an appropriate signal is given, a process referred to as adipogenesis (2). Changes in the expression levels of roughly 300 proteins are estimated to occur in the structural and functional morphogenesis associated with adipocyte differentiation (3).

Adipocyte differentiation is regulated by a number of hormones, growth factors, and cytokines. Essential components include cAMP, insulin, and glucocorticoids, while tumor necrosis factor (TNF)- $\alpha$ , a product of adipocytes, is known to inhibit differentiation (2,4–6). Differentiation can be characterized by chronological changes in the expression of numerous genes. These include lipoprotein lipase (LPL), peroxisome proliferator-activated receptor (PPAR)- $\gamma$ , GLUT4, glycerol-3-phosphate dehydrogenase (G3PDH), and leptin, which appear during different stages of differentiation, leading to the characteristic changes in morphology and the accumulation of triglyceride in the cytoplasm (2,5).

Leptin, the 167-amino acid product of the *ob* gene, is synthesized primarily in adipocytes and is a major regulator of fat and energy storage due to its effects on the hypothalamus and neuropeptide Y secretion. The leptin receptor is, however, widespread and is expressed on inflammatory blood cells, lung, liver, and intestine, and leptin has inhibitory effects on glucose metabolism and insulin secretion due to its effects on pancreatic  $\beta$ -cells (7). In obesity, there is leptin resistance to the elevated circulating leptin levels (7,8). Leptin has been shown in several studies to be an independent risk factor for coronary heart disease (9,10), but in obesity, sensitivity to effects on the sympathetic nervous system may not be decreased compared with metabolic effects. This may explain the hypertension observed in obesity (8).

Transcription factors play an important role in adipocyte differentiation. One important transcription factor is PPAR- $\gamma$ , which, in gain-of-function studies where nonadipogenic fibroblastic cells were made to express PPAR- $\gamma$ , a strong differentiation response was obtained (11,12). The use of synthetic ligands for PPAR- $\gamma$  such as the thiazolidinediones has shown promotion of adipogenesis in vitro (13), although the requirement for insulin, dexamethasone, and cAMP in vitro remains (14). However, PPAR- $\gamma$  activation inhibits leptin gene transcription in 3T3-L1 cells, and human studies show a reduction in circulating leptin with thiazolidinedione treatment (15,16). Autocrine TNF- $\alpha$  also inhibits leptin transcription in human adipose cells and inhibits other aspects of differentiation, such as

G3PDH, GLUT4, and PPAR- $\gamma$  expression. TNF- $\alpha$  also causes the dedifferentiation of human mature adipocytes (17,18).

This study aims to determine whether the differentiation program in adipocytes is differentially susceptible to inhibition by specific inhibitors of signaling pathways and PPAR- $\gamma$  activation. To accomplish this, we have treated differentiating human preadipocytes with four different inhibitor compounds: rapamycin, PD98059, wortmannin, and SB203580, which inhibit p70<sup>S6</sup> kinase, p42/44 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and p38 MAPK, respectively. Rapamycin inhibits the insulin-responsive kinase, p70<sup>S6</sup> kinase, by binding with FK-506-binding protein-12 (FKBP12), and this complex interacts with mTOR, causing the inhibition of p70<sup>S6</sup> kinase (19). PD98059 has been shown to inhibit MEK1, an enzyme that activates p42/44 MAPK (20). p42/44 MAPKs play a major role in the regulation of cell growth and differentiation in adipocytes and elsewhere (21). SB203580 is a pyridinyl imidazole that inhibits p38 MAPK (22), while wortmannin inhibits PI3K, an enzyme that selectively phosphorylates the 3-position of the inositol ring and is acutely activated by insulin and other growth factors (23). These compounds inhibit pathways known to be involved in cell differentiation. We have also examined the effect of PPAR- $\gamma$  activation on differentiation and on the effects of the inhibitors.

## RESEARCH DESIGN AND METHODS

**Subjects.** Subcutaneous adipose tissue was obtained from patients undergoing elective surgery in accordance with the guidelines of the South Birmingham ethics committee. None of the patients had diabetes or severe systemic illness, and none was taking medications known to influence adipose tissue mass, distribution, or metabolism.

**Isolation and culture of human preadipocytes.** Adipocytes were isolated by a variation of the method of Rodbell (24). Adipose tissue was digested with 1 mg/ml type I collagenase (Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution (HBSS), for 1 h at 37°C, and shaken at 100 cycles/min. The disrupted tissue was filtered through a double-layered cotton mesh, and isolated cells were washed with HBSS and centrifuged at 250g for 5 min to give a pellet containing preadipocytes. The cell pellet was resuspended in erythrocyte lysis buffer (154 mmol/l NH<sub>4</sub>Cl, 5.7 mmol/l K<sub>2</sub>HPO<sub>4</sub>, and 0.1 mmol/l EDTA, pH 7.0) for 10 min and centrifuged at 250g for 5 min to remove erythrocyte contamination. The resulting pellet was washed in HBSS, centrifuged at 250g for 5 min, and resuspended in DMEM/Ham's F-12 medium (Invitrogen, Paisley, U.K.) supplemented with 15% bovine FCS (First Link, Brierley Hill, U.K.). The cells were plated in tissue culture dishes and grown until confluent. All media used was supplemented with 100 units/ml penicillin G (Sigma) and 0.1 mg/ml streptomycin sulfate (Sigma).

**Differentiation of human preadipocytes.** Confluent preadipocytes were washed twice with HBSS and cultured in differentiation medium containing 100 nmol/l insulin (Sigma), 100 nmol/l dexamethasone (Dex) (Sigma), 0.2 nmol/l triiodothyronine (T3) (Sigma), and, for the first 4 days of culture, 25  $\mu$ mol/l 3-isobutyl-1-methylxanthine (IBMX) (Sigma). Cells were treated with four different inhibitor compounds, 10<sup>-7</sup> mol/l rapamycin (Calbiochem, Nottingham, U.K.), 10<sup>-5</sup> mol/l PD98059 (Calbiochem), 10<sup>-7</sup> mol/l wortmannin (Alexis, San Diego, CA), 10<sup>-6</sup> mol/l SB203580 (supplied by GlaxoSmithKline, U.K.) as well as the insulin-sensitizing agent 10<sup>-6</sup> mol/l rosiglitazone (supplied by GlaxoSmithKline, U.K.). Stock solutions were made up in DMSO, and control cultures were treated with differentiation medium and the vehicle DMSO. Cells were incubated in 5% CO<sub>2</sub>/95% air at 37°C, and differentiation medium and treatments were changed every 2–3 days for 20 days until cells had accumulated visible lipid droplets.

**Lipogenesis assay.** Lipogenesis was measured in 20-day-old differentiated preadipocytes using a variation of the method described by Moody et al. (25). Briefly cells were washed in HBSS and incubated overnight at 37°C, 5% CO<sub>2</sub>/95% air, in DMEM (GibcoBRL Life Technology) containing 5 mmol/l glucose (Sigma), 100 nmol/l insulin, 100 nmol/l Dex, 0.2 nmol/l T3, and 2  $\mu$ Ci/ml D-[U-<sup>14</sup>C]glucose (specific activity 291 mCi/mmol) (Amersham International, Slough, U.K.) as well as treatments with 10<sup>-7</sup> mol/l rapamycin, 10<sup>-5</sup>

mol/l PD98059, 10<sup>-7</sup> mol/l wortmannin, 10<sup>-6</sup> mol/l SB203580, and 10<sup>-6</sup> mol/l rosiglitazone. Cells were washed twice with HBSS, lipid was extracted using ethanol, and D-[U-<sup>14</sup>C]glucose radioactivity was determined by liquid scintillation counting.

**Lipid staining of differentiated preadipocytes.** Lipid staining was carried out using a variation of the method described by Culling (26). Briefly cells were washed with HBSS and stained with 2.5% Oil Red O dissolved in isopropanol (Sigma) for 15 min at room temperature. Cells were briefly washed with 60% isopropanol (Fisher Scientific U.K., Loughborough, U.K.) at room temperature and then washed twice with distilled water. Cells were viewed under a light microscope. Lipid and Oil Red O were extracted using ethanol, and the absorbance was measured on a spectrophotometer at 520 nm wavelength.

**Protein assay.** The cell layer was dissolved in sample buffer containing 2% sodium dodecyl sulfate (SDS) solution (2% SDS, 62.5 mmol/l Tris-HCl, pH 6.8), and protein content was determined using a modified Lowry assay (BioRad, Preston, U.K.).

**Leptin enzyme-linked immunosorbent assay.** Conditioned medium was collected from differentiated preadipocytes daily for a period of 12 days. The total amount of secreted leptin during differentiation was measured using an enzyme-linked immunosorbent assay (ELISA) kit (DRG Diagnostics, Marburg, Germany). The procedure was modified by the addition of 0.1% Triton added to samples and standards. Conditioned medium was not diluted, 50  $\mu$ l of sample was assayed directly, and standards were diluted in differentiation medium. These samples remained on the linear scale of the standard curve throughout the collection period.

**Determination of DNA content.** Nuclei of cells fixed in 95% ethanol were stained with acridine orange (10  $\mu$ g/ml in HBSS) (Sigma) for 1 min in the dark at room temperature. Cells were washed twice with HBSS, and the cell layer was taken up in 1 mol/l NaOH. DNA content was determined by measuring the excitation and emission wavelength at 485 nm and 535 nm in a fluorescence microplate reader.

**Western blot analysis for phosphorylated p38 MAPK.** Cell-layer proteins isolated from preadipocytes and differentiated preadipocytes treated with TNF- $\alpha$  (100 ng/ml) (PeproTechEC, London, U.K.) and dissolved in SDS solution were separated by SDS-PAGE using a 12.5% polyacrylamide gel and a 7.5% stack. The cell layer was dissolved in sample buffer, reduced with 10%  $\beta$ -mercaptoethanol, heated for 5 min at 95°C, and run on a gel. Prestained molecular weight markers (Sigma) were used as standards. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) hybrid membrane (Amersham International) by electroblotting at 425 mA for 3 h in a vertical transfer apparatus. The membrane was blocked by incubating in 10% nonfat milk in Tris-buffered saline-Tween 20 (TBS-T) (10 mmol/l Tris-HCl, pH 7.5, 100 mmol/l NaCl, 0.1% Tween 20) for 1 h at room temperature to prevent nonspecific binding. The membrane was incubated in anti-phospho-p38 MAPK primary antibody (New England Biolabs, Hertfordshire, U.K.) at a dilution of 1:1,000 in TBS-T with 10% nonfat milk for 3 h at room temperature. The membrane was washed three times in TBS-T and incubated with anti-rabbit IgG horseradish peroxidase (HRP) secondary antibody (Binding Site, Birmingham, U.K.) at a dilution of 1:10,000 in TBS-T for 1 h at room temperature. The antigens were detected by the enhanced chemiluminescence (ECL) system (Insight Biotechnology, Middlesex, U.K.) after exposure to X-ray film for 15 min.

**Statistics.** All experiments in the study were performed using adipose tissue from at least three patients ( $n \geq 3$ ). One-way ANOVA and unpaired Student's *t* test were used for data analysis in this study. Data are shown as means  $\pm$  SE. *P* values <0.05 were considered significant.

## RESULTS

**Differences in preadipocyte differentiation in the presence of rosiglitazone measured by cell morphology.** Preadipocytes were induced to differentiate for 20 days in medium containing insulin, Dex, T3, and, for the first 4 days of culture, IBMX. Figure 1A shows preadipocytes before differentiation is induced. The preadipocytes have a characteristic fibroblastic appearance. When differentiation is induced, morphological changes occur with cells becoming spherical with cytoplasmic lipid droplets accumulating as shown in Fig. 1B. The addition of rosiglitazone to the differentiation medium markedly increased the number of visible lipid droplets as shown in Fig. 1C compared with preadipocytes differentiated in differentiation medium alone shown in Fig. 1B.

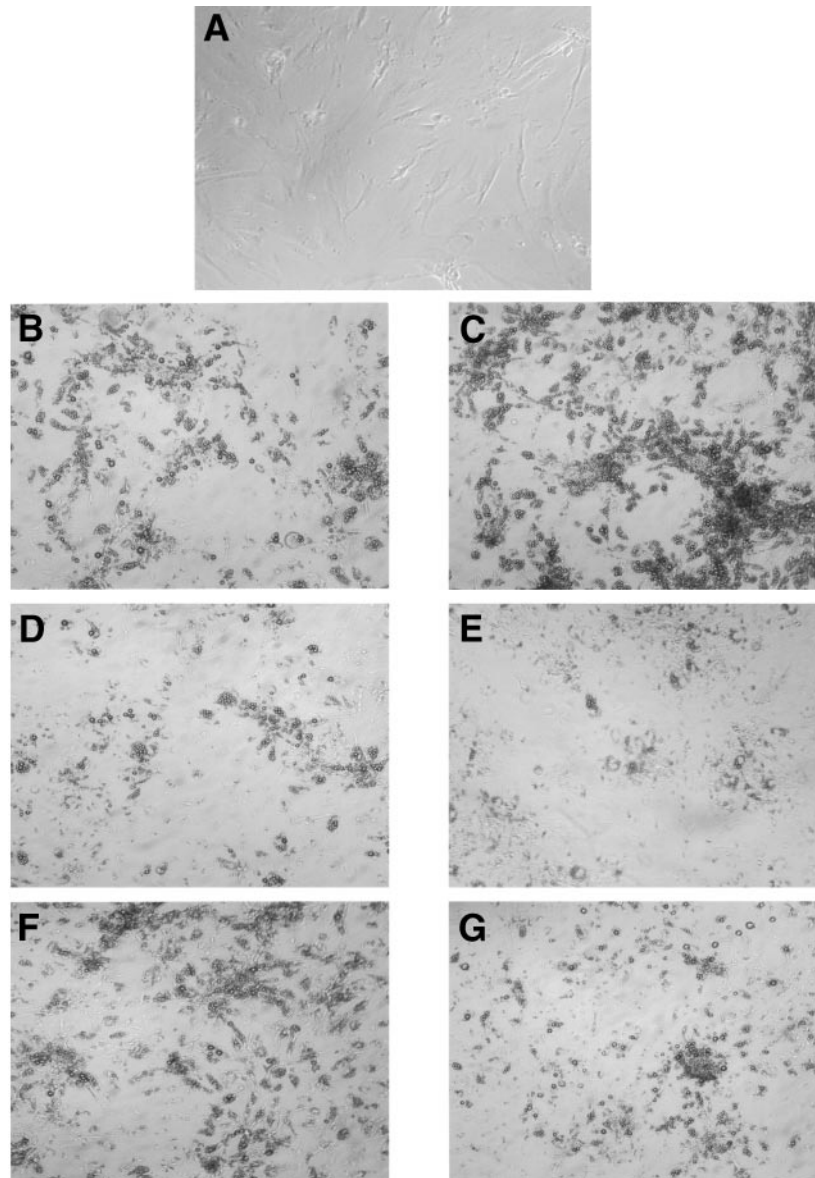


FIG. 1. The effect of rosiglitazone and the inhibitors on adipocyte differentiation. *A*: Human preadipocytes. *B*: Twenty-day culture of preadipocytes in differentiation medium. *C*: Twenty-day culture of preadipocytes in differentiation medium with rosiglitazone ( $10^{-6}$  mol/l). *D*: Twenty-day culture of preadipocytes in differentiation medium and rapamycin ( $10^{-7}$  mol/l). *E*: Twenty-day culture of preadipocytes in differentiation medium and SB203580 ( $10^{-6}$  mol/l). *F*: Twenty-day culture of preadipocytes in differentiation medium and PD98059 ( $10^{-5}$  mol/l). *G*: Twenty-day culture of preadipocytes in differentiation medium and wortmannin ( $10^{-7}$  mol/l). Magnification  $\times 100$ .

**Effects of rosiglitazone on cell survival, leptin secretion, lipid accumulation, and lipogenesis.** To assess the effect of rosiglitazone on the differentiation, five different parameters were examined. These were protein content, total leptin secretion over 12 days of differentiation, total DNA content, the amount of accumulated lipid, and lipogenesis. The final two parameters have been corrected for protein content. Table 1 shows the results from a representative experiment. This experiment was repeated 15 times with comparable data. Relative effects were maintained, although individual preparations show variation in their extent of differentiation (27). The addition of rosiglitazone to the differentiation medium caused a significant increase of 15% in protein content of the cells ( $P < 0.01$ ). DNA content was increased by a similar amount, although the effect was not statistically significant. Rosiglitazone increased the amount of lipid accumu-

lated twofold ( $P < 0.0001$ ), and the level of de novo lipogenesis was increased sevenfold ( $P < 0.0001$ ) when compared with cells cultured for 20 days in differentiation medium alone. Rosiglitazone had no effect on the total amount of leptin secreted over 12 days of preadipocyte differentiation compared with cells cultured for 12 days in differentiation medium alone.

**Effects of rapamycin, SB203580, PD98059, and wortmannin inhibitors on protein synthesis of differentiated preadipocytes.** Preadipocytes were induced to differentiate over 20 days in differentiation medium with and without rosiglitazone. Four inhibitor compounds, rapamycin, SB203580, PD98059, and wortmannin, were added to cells when differentiation was initiated and were maintained in the medium throughout the 20-day period. After this 20-day period, the cell layer was removed and the amount of protein present was determined. None of



TABLE 1  
The effect of rosiglitazone on adipocyte differentiation

Assay	Differentiation medium	Differentiation medium + rosiglitazone	P
Protein content (mg/ml)	0.70 ± 0.00	0.85 ± 0.02	<0.01
Leptin content (ng/ml)	10.52 ± 3.09	8.22 ± 0.74	>0.05
DNA content (485:535nm) arbitrary units × 10 <sup>-6</sup>	2.04 ± 0.10	2.36 ± 0.17	>0.05
Lipid content (Abs 520 nm)/protein content (mg/ml)	1.21 ± 0.10	2.43 ± 0.00	<0.0001
<sup>14</sup> C-lipid (dpm × 10 <sup>-3</sup> )/protein content (mg/ml)	4.01 ± 0.35	28.97 ± 1.43	<0.0001

Data are means ± SE. Preadipocytes were cultured in differentiation medium and differentiation medium containing rosiglitazone for 20 days. After this period, the protein content, leptin content, cell count, lipid content, and the level of lipogenesis were assessed to determine the level of differentiation. The data shown are a representative experiment, and the lipid content and lipogenesis data have been corrected for protein content.

the inhibitors had a significant effect on the total amount of protein present after 20 days of differentiation (data not shown).

**Effects of rapamycin, SB203580, PD98059, and wortmannin inhibitors on lipid accumulation of differentiated preadipocytes.** To assess the degree of differentiation, lipid accumulation in differentiated cells was measured. Figure 1B shows 20-day-old differentiated preadipocytes, while Fig. 1D-G shows 20-day-old differentiated preadipocytes treated with rapamycin, SB203580, PD98059, and wortmannin, respectively. The addition of rapamycin and SB203580 (Fig. 1D and E) significantly reduced the amount of spherical lipid droplets observed while the addition of PD98059 and wortmannin (Fig. 1F and G) had little effect on lipid accumulation compared with differentiated preadipocytes without inhibitor (Fig. 1B).

To quantify this effect on lipid accumulation, the 20-day-old preadipocytes were stained with the dye Oil Red O, and the amount of stain taken up was quantified. Figure 2 shows the effect of the inhibitors on lipid accumulation of differentiated cells in the presence and absence of rosiglitazone in the differentiation medium. Rapamycin (74% of control,  $P < 0.05$ ) and SB203580 (77% of control,  $P < 0.05$ ) significantly reduced the level of lipid accumulation in

cells, while PD98059 and wortmannin had no significant effect (Fig. 2). In preadipocytes differentiated in the presence of rosiglitazone, rapamycin (63% of control,  $P < 0.01$ ) and SB203580 (52% of control,  $P < 0.01$ ) again significantly inhibited lipid accumulation, while PD98059 and wortmannin had no effect (Fig. 2).

**Effects of rapamycin, SB203580, PD98059, and wortmannin inhibitors on lipogenesis of differentiated preadipocytes.** To determine lipogenesis, the uptake of <sup>14</sup>C-glucose in 20-day-old differentiated preadipocytes was assessed. Cells had been differentiated with and without rosiglitazone in the differentiation medium, and the inhibitors had been added to the cells throughout the 20-day period. Rapamycin (75% of control,  $P < 0.05$ ) and SB203580 (53% control,  $P < 0.01$ ) significantly reduced the uptake of <sup>14</sup>C-glucose into lipid, while PD98059 and wortmannin had no significant effect (Fig. 3). In preadipocytes differentiated in the presence of rosiglitazone, rapamycin (67% of control,  $P < 0.01$ ) and SB203580 (36% of control,  $P < 0.01$ ) again significantly reduced the incorporation of <sup>14</sup>C-glucose into lipid, while PD98059 and wortmannin had no effect (Fig. 3). The inhibitory effects were enhanced in the presence of rosiglitazone.

**Effects of rapamycin, SB203580, PD98059, and wortmannin inhibitors on leptin secretion from differen-**

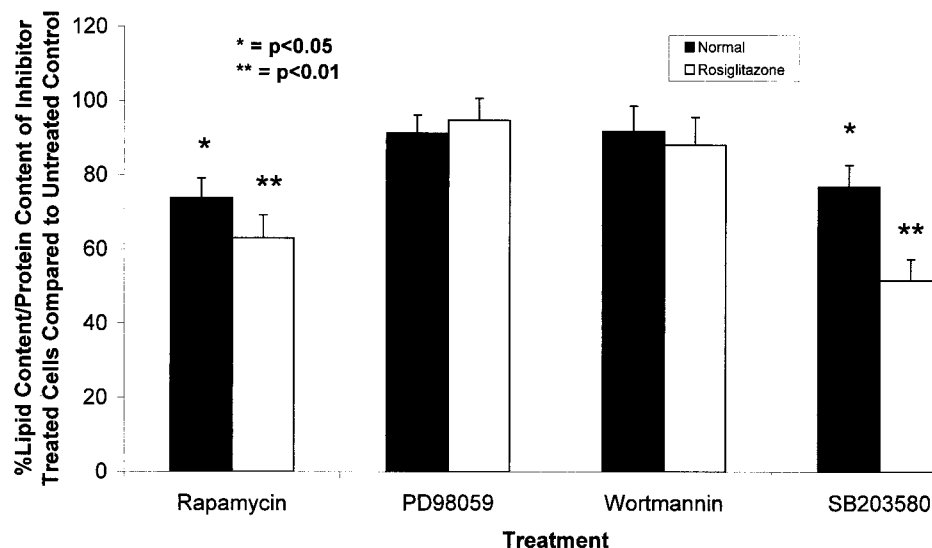


FIG. 2. The effect of the four inhibitors on the lipid content of differentiated preadipocytes with and without rosiglitazone (10<sup>-6</sup> mol/l). After 20 days, lipid content was measured as described in RESEARCH DESIGN AND METHODS. Results are given as the percentage mean value compared with untreated control differentiated preadipocytes and are corrected for protein content ± SE of four independent samples ( $n = 4$ ).

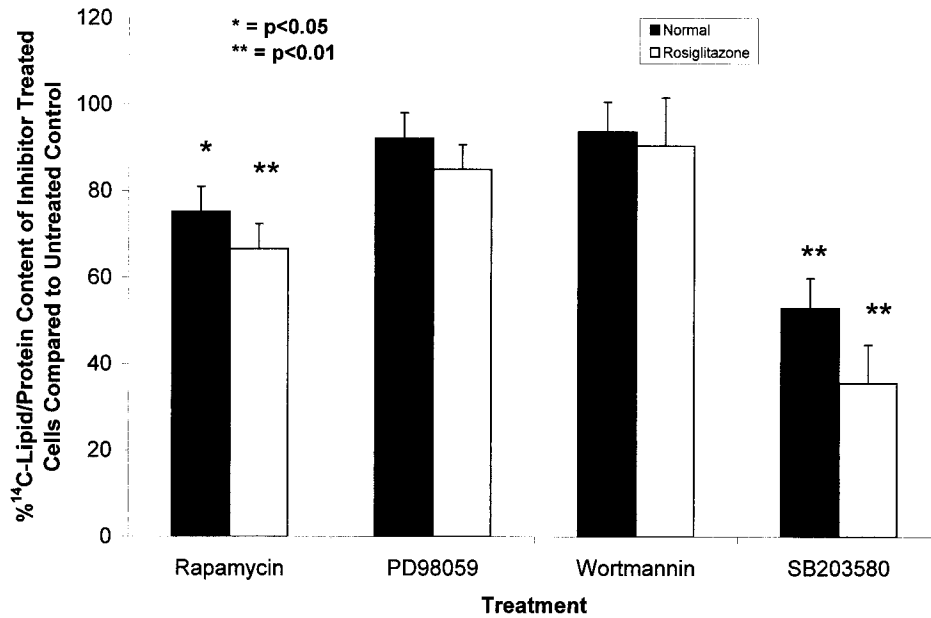


FIG. 3. The effect of the four inhibitors on lipogenesis of differentiated preadipocytes with and without rosiglitazone ( $10^{-6}$  mol/l). After 20 days, lipogenesis was measured as described in RESEARCH DESIGN AND METHODS. Results are given as the percentage mean value compared with untreated control differentiated preadipocytes and are corrected for protein content  $\pm$  SE of four independent samples ( $n = 4$ ).

**tiated preadipocytes.** Preadipocytes were differentiated in the presence of the four inhibitors rapamycin, SB203580, PD98059, and wortmannin over 12 days, and the conditioned medium was collected daily. To determine the total amount of leptin secreted, an ELISA was carried out on the conditioned medium collected over 12 days, allowing the determination of total leptin secreted after 12 days of differentiation.

Figure 4 shows the effect of the four inhibitors on leptin secretion during differentiation over a 12-day period. The addition of SB203580 (6% of control,  $P < 0.01$ ) markedly

inhibited leptin secretion, while the addition of wortmannin (22% of control,  $P < 0.05$ ), rapamycin (34% of control,  $P < 0.05$ ), and PD98059 (34% of control,  $P < 0.05$ ) also significantly inhibited leptin secretion compared with control.

**Effect of TNF- $\alpha$  treatment on phospho-p38 MAPK.** Preadipocytes and differentiated preadipocytes were treated with TNF- $\alpha$  (100 ng/ml) for different time periods. Figure 5A shows the time course of phospho-p38 MAPK activation in preadipocytes, while Fig. 5B shows the time course of phospho-p38 MAPK activation in differentiated

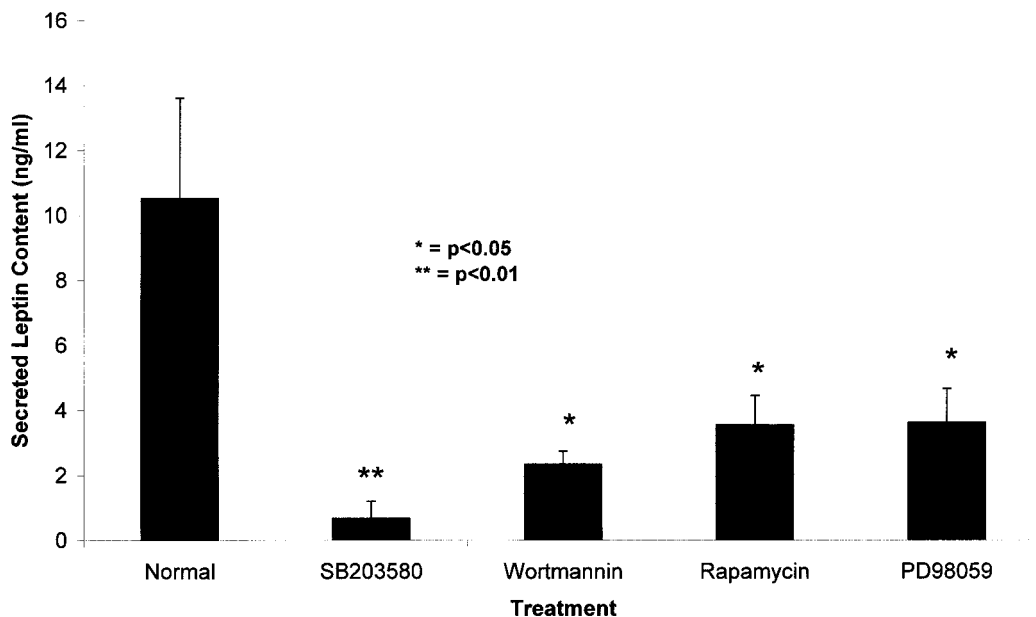
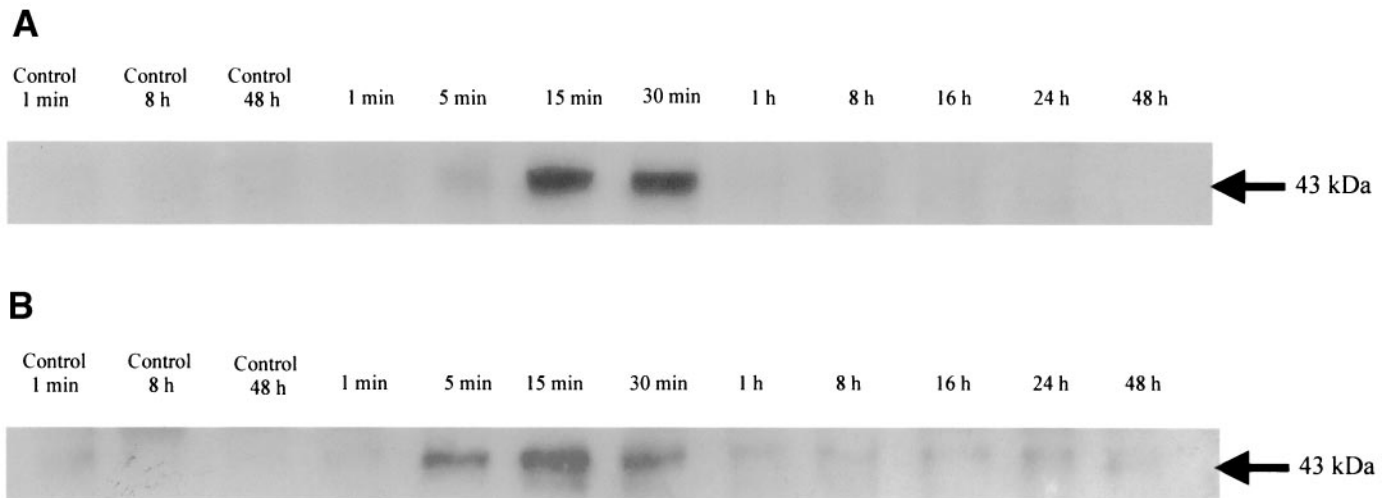


FIG. 4. The effect of the four inhibitors on the secreted leptin content of differentiated preadipocytes. Preadipocytes were differentiated over a 12-day period with the inclusion of the inhibitors. Conditioned medium was removed daily and the secreted leptin content was measured using an ELISA kit, allowing the total secreted leptin content of cells differentiated over 12 days to be determined. Results are given as the mean  $\pm$  SE of three independent samples ( $n = 3$ ).



**FIG. 5.** The effects of TNF- $\alpha$  treatment on phospho-p38 MAPK activation. **A:** Preadipocytes were cultured in DMEM:F12 with TNF- $\alpha$  (100 ng/ml) for indicated incubation periods. Control lanes contain no TNF- $\alpha$  treatment. Phospho-p38 MAPK activation was detected in the cell layer by Western blotting. **B:** Twenty-day-old differentiated preadipocytes were cultured in DMEM:F12 with TNF- $\alpha$  (100 ng/ml) for indicated incubation periods. Control lanes contain no TNF- $\alpha$  treatment. Phospho-p38 MAPK activation was detected in the cell layer by Western blotting.

preadipocytes. A single band of 43 kDa was detectable after 5 min incubation with TNF- $\alpha$  in preadipocytes (Fig. 5A) and 15 min TNF- $\alpha$  incubation in differentiated preadipocytes (Fig. 5B). After 1 h, phospho-p38 MAPK levels reduced to undetectable levels.

## DISCUSSION

In human preadipocyte differentiation, we found that rosiglitazone increased the protein and DNA content of the cell layer modestly. Lipid content of cells was increased twofold but lipogenesis was increased sevenfold compared with cells differentiated in differentiation medium without rosiglitazone. In contrast, leptin secretion was not increased with rosiglitazone treatment, consistent with a preferential inhibition of this marker of differentiation compared with lipogenesis. Studies with 3T3 cells (15) and short-term *in vitro* studies in mature human adipocytes showed that PPAR- $\gamma$  activation reduces leptin mRNA. Our data show that throughout differentiation PPAR- $\gamma$  activation increases the extent and rate of differentiation into mature fat cells while having no such stimulatory effect on leptin synthesis. Consistent with its role as an insulin sensitizer, PPAR- $\gamma$  activation in human adipocytes was found to increase the transcription and insulin-induced activation of the p85 $\alpha$  subunit of PI3K (28). Since insulin is reported to be a repressor of glucocorticoid-induced leptin synthesis (29), as well as having antilipolytic functions (30), our studies showing no increase in leptin secretion compared with elevated lipid stores following PPAR- $\gamma$  activation are compatible with these data.

We used specific inhibitors of different signaling pathways and assessed the effect on markers of adipocyte differentiation. Inhibition of p70<sup>S6</sup> kinase and p38 MAPK inhibited adipocyte differentiation, assessed by the lipogenic activity of differentiated cells and the level of lipid accumulation within the cells. The inhibitory effects were greater for p38 MAPK inhibition. PI3K and p42/44 MAPK inhibition, however, had no inhibitory effect on lipid metabolism, but all four inhibitors markedly inhibited leptin secretion, a late marker for adipocyte differentiation.

p70<sup>S6</sup> kinase has been implicated in protein synthesis, especially in the transcriptional and translational regulation of insulin effects (31). We did not find inhibitory effects on protein synthesis over 20 days in culture, suggesting that rapamycin is not cytotoxic. However, new protein synthesis, which would be necessary to initiate a program of differentiation, may be limited.

In agreement with studies in 3T3-L1 cells (32), we found that p38 MAPK inhibition inhibits human adipocyte differentiation. Its essential roles in inflammation, growth, differentiation, the cell cycle, and cell death have been shown in other cell types (22). In 3T3-L1 cells, the active phosphorylated form of the transcription factor CCAAT/enhancer-binding protein (C/EBP)  $\beta$  was reduced by SB203580 (32). C/EBP  $\beta$  is an activator of C/EBP  $\alpha$  and PPAR- $\gamma$ , two potent adipogenic transcription factors (33). Inhibition of p38 MAPK may thus inhibit C/EBP  $\alpha$  and PPAR- $\gamma$  activation and block adipocyte differentiation (32) in this way.

We show here that TNF- $\alpha$  activates the p38 MAPK pathway in human preadipocytes and adipocytes consistent with data from 3T3-L1 cells (32) and fetal brown adipocytes (34). Because our data show that the p38 MAPK pathway is essential for differentiation, we conclude that the inhibitory effects of TNF- $\alpha$  on differentiation are not mediated by the p38 MAPK signaling pathway.

The noninvolvement of the p42/44 MAPK signaling pathway in adipocyte lipogenesis has been shown in some, but not all, studies with 3T3-L1 cells (4). In human cells, PPAR- $\gamma$  is a substrate for p42/44 MAPK, and its phosphorylation leads to decreased adipocyte differentiation (6,35,36). A genetic mutation in PPAR- $\gamma$  at this phosphorylation site has been found in obese individuals (37). Blocking p42/44 MAPK should therefore increase differentiation, but our studies in human adipocytes induced to differentiate in long-term culture show that other pathways downstream of p42/44 MAPK must mitigate this stimulation.

PI3K plays an essential role in the regulation of various cellular activities, including proliferation, differentiation,

and the prevention of apoptosis (38). In this study, we show that the inhibition of PI3K by wortmannin had no effect on human adipocyte lipogenesis. This contradicts the results of other studies, which have shown that wortmannin inhibits adipocyte differentiation (38,39). However, those studies have been carried out in cell lines such as 3T3-L1 cells where postconfluent mitoses are thought to be required early on for successful differentiation of this cell line (clonal expansion phase) (40). PI3K inhibition is likely to interfere with this phase, whereas this staging is not required in human adipocytes. Studies looking at insulin treatment on GLUT4 levels have shown differences between 3T3-F442A and primary rat adipocytes, thus reinforcing the apparent differences between cell lines and primary culture (41), and Ryden et al. (42) found constitutively active p42/44 MAPK in these cells. It is surprising that wortmannin is less potent than rapamycin in our assays, because PI3K is upstream of the rapamycin target p70<sup>S6</sup> kinase. Our data suggest that the pathways are not directly linked.

Insulin has a known antilipolytic action, and it was possible that the antilipolytic effect would be separable from stimulatory effects on lipogenesis. However, with all of our inhibitors, the effects on lipogenesis are directly comparable to the effects on lipid content in the cells. This suggests that the antilipolytic effects of insulin are not mediated through these pathways. For rosiglitazone, stimulatory effects were more marked on lipogenesis than lipid accumulation, suggesting preferential effects.

In contrast to the disparate effects on lipogenesis, all four inhibitors profoundly inhibited leptin secretion. PD98059 and wortmannin, which inhibit pathways involved in insulin signaling, inhibited leptin secretion without inhibition of lipogenesis and lipid storage. Since insulin stimulates secretion of stores of leptin and does not increase leptin expression (43), it seems unlikely that its effects on leptin gene expression are mediated through these pathways. However, it should be remembered that the design of this study differs from the acute, short-term studies showing insulin-mediated inhibition of leptin mRNA (44,45). Indirect effects of insulin on genes important in leptin gene transcription may occur. These could include autocrine growth factors such as fibroblast growth factors, insulin-like growth factors (IGFs), and angiopoietins, which will use some of these signaling pathways. Furthermore, dexamethasone and cortisol are known to increase leptin expression and to interact with the MAPK signaling pathway. Steroid receptors recruit p160 family members whose activity is regulated by MAPKs (46). Inhibition of MAPK may thus inhibit glucocorticoid receptor signaling and inhibit leptin production (43).

Serum leptin has been shown to be an independent risk factor in cardiovascular disease (9,10). Both insulin and rosiglitazone increase differentiation of preadipocytes and increase adipose mass. Inhibiting different signaling pathways resulted in inhibition of leptin secretion, but not always inhibition of adipocyte differentiation. This suggests that there is a redundancy in signaling as far as differentiation is concerned, but not in the regulation of leptin secretion. Rosiglitazone treatment is associated with favorable changes in adipose tissue cytokines, including leptin, adiponectin, and TNF- $\alpha$  (16,47,48). Our data

indicate that this is due to stimulatory effects of PPAR- $\gamma$  agonists on lipogenesis as opposed to leptin secretion.

#### ACKNOWLEDGMENTS

This work was supported by the Medical Research Council CASE award (Grant no. RURC 6439) and GlaxoSmithKline U.K. We thank all the theater staff at the Priory Hospital, Edgbaston, who aided in these studies. We also thank Mr. Levick for kindly providing consented samples from his plastic surgery operations at the Priory Hospital.

#### REFERENCES

- Pi-Sunyer FX: The medical risks of obesity. *Obes Surg* 12 (Suppl. 1):6S-11S, 2002
- Sorisky A: From preadipocyte to adipocyte: differentiation-directed signals of insulin from the cell surface to the nucleus. *Crit Rev Clin Lab Sci* 36:1-34, 1999
- Morrison RF, Farmer SR: Hormonal signaling and transcriptional control of adipocyte differentiation. *J Nutr* 130:3116S-3121S, 2000
- Font de Mora J, Porras A, Ahn N, Santos E: Mitogen-activated protein kinase activation is not necessary for, but antagonizes, 3T3-L1 adipocyte differentiation. *Mol Cell Biol* 17:6068-6075, 1997
- Gregoire FM, Smas CM, Sul HS: Understanding adipocyte differentiation. *Physiol Rev* 78:783-809, 1998
- Camp HS, Tafuri SR: Regulation of peroxisome proliferator-activated receptor gamma activity by mitogen-activated protein kinase. *J Biol Chem* 272:10811-10816, 1997
- Ahima RS, Flier JS: Leptin. *Annu Rev Physiol* 62:413-437, 2000
- Rahmouni K, Haynes WG, Mark AL: Cardiovascular and sympathetic effects of leptin. *Curr Hypertens Rep* 4:119-125, 2002
- Wallace AM, McMahon AD, Packard CJ, Kelly A, Shepherd J, Gaw A, Sattar N: Plasma leptin and the risk of cardiovascular disease in the west of Scotland coronary prevention study (WOSCOPS). *Circulation* 104:3052-3056, 2001
- Soderberg S, Ahren B, Jansson JH, Johnson O, Hallmans G, Asplund K, Olsson T: Leptin is associated with increased risk of myocardial infarction. *J Intern Med* 246:409-418, 1999
- Tontonoz P, Hu E, Spiegelman BM: Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell* 79:1147-1156, 1994
- Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM: Transcriptional regulation of adipogenesis. *Genes Dev* 14:1293-1307, 2000
- Adams M, Montague CT, Prins JB, Holder JC, Smith SA, Sanders L, Digby JE, Sewter CP, Lazar MA, Chatterjee VK, O'Rahilly S: Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentiation. *J Clin Invest* 100:3149-3153, 1997
- Grimaldi PA: The roles of PPARs in adipocyte differentiation. *Prog Lipid Res* 40:269-281, 2001
- Kallen CB, Lazar MA: Antidiabetic thiazolidinediones inhibit leptin (ob) gene expression in 3T3-L1 adipocytes. *Proc Natl Acad Sci U S A* 93:5793-5796, 1996
- Shimizu H, Tsuchiya T, Sato N, Shimomura Y, Kobayashi I, Mori M: Troglitazone reduces plasma leptin concentration but increases hunger in NIDDM patients. *Diabetes Care* 21:1470-1474, 1998
- Zhang HH, Kumar S, Barnett AH, Eggo MC: Tumour necrosis factor-alpha exerts dual effects on human adipose leptin synthesis and release. *Mol Cell Endocrinol* 159:79-88, 2000
- Petruschke T, Hauner H: Tumour necrosis factor-alpha prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. *J Clin Endocrinol Metab* 76:742-747, 1993
- Bell A, Grunder L, Sorisky A: Rapamycin inhibits human adipocyte differentiation in primary culture. *Obes Res* 8:249-254, 2000
- Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR: PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. *J Biol Chem* 270:27489-27494, 1995
- Aubert J, Belmonte N, Dani C: Role of pathways for signal transducers and activators of transcription, and mitogen-activated protein kinase in adipocyte differentiation. *Cell Mol Life Sci* 56:538-542, 1999
- Ono K, Han J: The p38 signal transduction pathway: activation and function. *Cell Signal* 12:1-13, 2000
- Evans JL, Honer CM, Womelsdorf BE, Kaplan EL, Bell PA: The effects of wortmannin, a potent inhibitor of phosphatidylinositol 3-kinase, on insulin-



- stimulated glucose transport, GLUT4 translocation, antilipolysis, and DNA synthesis. *Cell Signal* 7:365–376, 1995
24. Rodbell M: Metabolism of isolated fat cells: effect of hormones on glucose metabolism. *J Biol Chem* 239:375–380, 1964
  25. Moody AJ, Stan MA, Stan M, Gliemann J: A simple free fat cell bioassay for insulin. *Horm Metab Res* 6:12–16, 1974
  26. Culling CFA: *Handbook of Histopathological Techniques*. 2nd ed. London, Butterworth, 1963
  27. Sen A, Lea-Currie YR, Sujkowska D, Franklin DM, Wilkison WO, Halvorsen YD, Gimble JM: Adipogenic potential of human adipose derived stromal cells from multiple donors is heterogeneous. *J Cell Biochem* 81:312–319, 2001
  28. Rieusset J, Auwerx J, Vidal H: Regulation of gene expression by activation of the peroxisome proliferator-activated receptor gamma with rosiglitazone (BRL 49653) in human adipocytes. *Biochem Biophys Res Commun* 265:265–271, 1999
  29. Reul BA, Ongemba LN, Pottier AM, Henquin JC, Brichard SM: Insulin and insulin-like growth factor 1 antagonize the stimulation of ob gene expression by dexamethasone in cultured rat adipose tissue. *Biochem J* 324:605–610, 1997
  30. Rieusset J, Chambrier C, Bouzakri K, Dusserre E, Auwerx J, Riou JP, Laville M, Vidal H: The expression of the p85alpha subunit of phosphatidylinositol 3-kinase is induced by activation of the peroxisome proliferator-activated receptor gamma in human adipocytes. *Diabetologia* 44:544–554, 2001
  31. Berven LA, Crouch MF: Cellular function of p70S6K: a role in regulating cell motility. *Immunol Cell Biol* 78:447–451, 2000
  32. Engelman JA, Lisanti MP, Scherer PE: Specific inhibitors of p38 mitogen-activated protein kinase block 3T3–L1 adipogenesis. *J Biol Chem* 273:32111–32120, 1998
  33. Cowherd RM, Lyle RE, McGehee RE Jr: Molecular regulation of adipocyte differentiation. *Semin Cell Dev Biol* 10:3–10, 1999
  34. Valladares A, Alvarez AM, Ventura JJ, Roncero C, Benito M, Porras A: p38 mitogen-activated protein kinase mediates tumor necrosis factor-alpha-induced apoptosis in rat fetal brown adipocytes. *Endocrinology* 141:4383–4395, 2000
  35. Hu E, Kim JB, Sarraf P, Spiegelman BM: Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma. *Science* 274:2100–2103, 1996
  36. Zhang B, Berger J, Zhou G, Elbrecht A, Biswas S, White-Carrington S, Szalkowski D, Moller DE: Insulin-and mitogen-activated protein kinase-mediated phosphorylation and activation of peroxisome proliferator-activated receptor gamma. *J Biol Chem* 271:31771–31774, 1996
  37. Ristow M, Muller-Wieland D, Pfeiffer A, Krone W, Kahn CR: Obesity associated with a mutation in a genetic regulator of adipocyte differentiation. *N Engl J Med* 339:953–959, 1998
  38. Xia X, Serrero G: Inhibition of adipose differentiation by phosphatidylinositol 3-kinase inhibitors. *J Cell Physiol* 178:9–16, 1999
  39. Tomiyama K, Nakata H, Sasa H, Arimura S, Nishio E, Watanabe Y: Wortmannin, a specific phosphatidylinositol 3-kinase inhibitor, inhibits adipocytic differentiation of 3T3–L1 cells. *Biochem Biophys Res Commun* 212:263–269, 1995
  40. Yeh WC, Bierer BE, McKnight SL: Rapamycin inhibits clonal expansion and adipogenic differentiation of 3T3–L1 cells. *Proc Natl Acad Sci U S A* 92:11086–11090, 1995
  41. Buren J, Liu HX, Jensen J, Eriksson JW: Dexamethasone impairs insulin signalling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase B in primary cultured rat adipocytes. *Eur J Endocrinol* 146:419–429, 2002
  42. Ryden M, Dicker A, van Harmelen V, Hauner H, Brunnberg M, Perbeck L, Lonnqvist F, Arner P: Mapping of early signaling events in tumor necrosis factor-alpha-mediated lipolysis in human fat cells. *J Biol Chem* 277:1085–1091, 2002
  43. Bradley RL, Cheatham B: Regulation of ob gene expression and leptin secretion by insulin and dexamethasone in rat adipocytes. *Diabetes* 48:272–278, 1999
  44. Hardie LJ, Guillhot N, Trayhurn P: Regulation of leptin production in cultured mature white adipocytes. *Horm Metab Res* 28:685–689, 1996
  45. Gettys TW, Harkness PJ, Watson PM: The beta 3-adrenergic receptor inhibits insulin-stimulated leptin secretion from isolated rat adipocytes. *Endocrinology* 137:4054–4057, 1996
  46. Lopez GN, Turck CW, Schaufele F, Stallcup MR, Kushner PJ: Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity. *J Biol Chem* 276:22177–22182, 2001
  47. McTernan PG, Harte AL, Anderson LA, Green A, Smith SA, Holder JC, Barnett AH, Eggo MC, Kumar S: Insulin and rosiglitazone regulation of lipolysis and lipogenesis in human adipose tissue in vitro. *Diabetes* 51:1493–1498, 2002
  48. Combs TP, Wagner JA, Berger J, Doebber T, Wang WJ, Zhang BB, Tanen M, Berg AH, O'Rahilly S, Savage DB, Chatterjee K, Weiss S, Larson PJ, Gottesdiener KM, Gertz BJ, Charron MJ, Scherer PE, Moller DE: Induction of adipocyte complement-related protein of 30 kilodaltons by PPARgamma agonists: a potential mechanism of insulin sensitization. *Endocrinology* 143:998–1007, 2002